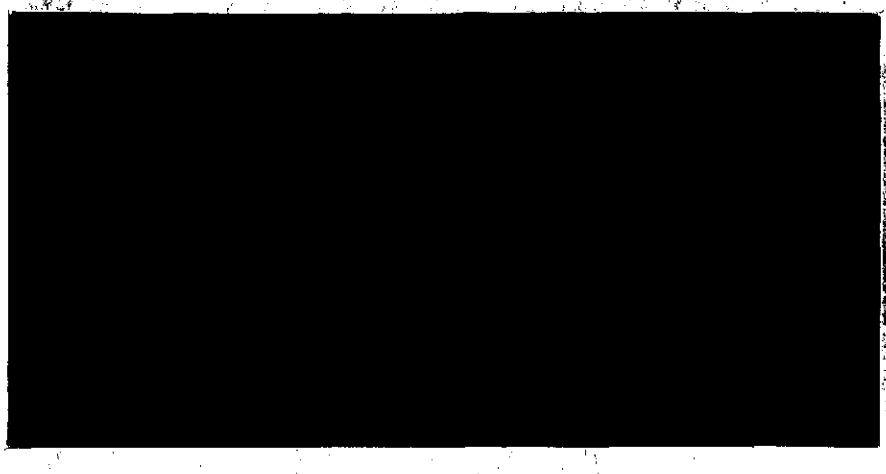
THE PERFORMANCE AND CAPABILITIES OF TERRESTRIAL ORGANISMS IN EXTREME AND UNUSUAL GASEOUS AND LIQUID ENVIRONMENTS



S. M. SIEGEL

BOTANY DEPARTMENT, UNIVERSITY OF HAW

Prepared under
Grant No. NGL 12-001-042
with the
NATIONAL AERONAUTICS AND SPACE ADMINISTRATION

SEMI-ANNUAL REPORT

THE PERFORMANCE AND CAPABILITIES OF TERRESTRIAL ORGANISMS IN EXTREME AND UNUSUAL GASEOUS AND LIQUID ENVIRONMENTS

Halo- and Cryotolerance of the Alga *Dunaliella*

November 1974

University of Hawaii
Hawaii Botanical Science Paper
No. 38

Informal technical report, neither to be cited nor credited as a publication.

Submitted by: S M Siegel

Sanford M. Siegel
Professor of Botany &
Principal Investigator
University of Hawaii
Honolulu, Hawaii 96822

NATIONAL AERONAUTICS AND SPACE ADMINISTRATION

Grant No. NGL 12-001-042

This dissertation is part of a research program supported by National Aeronautics and Space Administration Research Grant NGL 12-001-042.

THE CRYO- AND HALOBIOLOGY OF *DUNALIELLA*

Part I

A Dissertation submitted to the Graduate Division of the University of Hawaii in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Botanical Sciences

By

Thomas W. Speitel

TABLE OF CONTENTS

INTRODUCTION

The accounts of physiological research on the active metabolism and behavior of autotrophic eukaryotes at sub-zero temperatures are relatively meagre. Possibly, this is because so few organisms remain unfrozen at temperatures of 0°C to -15°C. This report will deal with the alga *Dunaliella*, and its remarkable cryo- and halo-tolerance.

The genus *Dunaliella*, composed of green unicellular algae, is in the family Polybelpharidaceae (phylum Chlorophyta) (24). The organisms are motile, with two equal, unipolar flagella. They possess a thin, elastic cell envelope which follows changes in body outline. Reproduction occurs by longitudinal fission or by fusion of two motile, haploid cells to form a zygote (136). This genus resembles *Chlamydomonas*, but differs in the nature of the cell envelope. *Chlamydomonas* has not only a periplast but also a cell wall. The movement of most species of *Dunaliella* is a constant, linear motion. The algae rotate only slowly, and when quiescent, continue to vibrate. *Dunaliella* is of commerical importance as a source of vitamin A (105, 107, 108, 109).

Dunaliella have been recorded in oceans, salt marshes, inland salt springs, lagoons and saline pools all over the world (24, 105, 170). Some specimens have been found at depths of 315 meters in the Dead Sea (44).

The similarities and differences in salt and cold tolerance mechanisms of *Dunaliella salina* and *Dunaliella tertiolecta* will be under focus in this investigation.

The NASA Technical Officer for this grant is D. S. Geib, Planetary Programs, NASA, Washington, D. C. 20546.

THE BIOLOGY OF *DUNALIELLA TERTIOLECTA* AND *DUNALIELLA SALINA*

D. salina and *D. tertiolecta* differ from each other in morphology. Although both species are ovoid or ellipsoid, mature *D. salina* cells usually have at least twice the volume of *D. tertiolecta* cells. The periplast of *D. salina* is less firm than that of *D. tertiolecta*. Both species have companulate chromatophores, *D. tertiolecta*'s being more rugose. The pyrenoid of *D. salina* is sub-basal and *D. tertiolecta*'s is central. *D. salina* contains a large number of refractive granules arranged in a girdle just above the chromatophore. The granules of *D. tertiolecta* are distributed evenly throughout the cell. *D. salina* has a haploid number of chromosomes of about 10, while the count for *D. tertiolecta* has not been recorded (24, 134).

Light optima for photosynthesis of both species have been reported. *D. salina* is positively phototaxic (17). Reports of optimal light intensities for growth range from 600 lux (116) to 10,000 lux (50, 170). *D. salina*'s carotene composition (63) and accumulation with higher salinities is documented (37, 38, 39, 40, 41, 42).

After high illumination, *D. tertiolecta* needs a short period in the dark to develop maximum photosynthetic rate (64). Oxygen evolution is higher with NH_3 than NO_3 as a nitrogen source (133). Grant (55) gave evidence for the existence of two independent systems of nitrate reduction, one within and the other outside

the chloroplast. The enzyme system which fixes CO_2 is likely to be the rate limiting step in photosynthesis. Glycerol is the major soluble photosynthate (31). Photopigment extractions and concentrations have been reported (34, 101, 138, 159, 163).

Redox enzymes of *D. salina* such as cytochrome oxidase, peroxidase, catalase, reductase-ascorbate oxidase and polyphenol oxidase have been studied. Its polyphenol oxidase is specific for triphenols (122). For *D. tertiolecta*, nitrate reductase (56), glucose 6-phosphate dehydrogenase, pyrophosphatase, ATP-ase (75), hexokinase (89), ribulose di-phosphate carboxylase and aldolase (64, 133) have been studied.

The general mechanism of salt tolerance for *D. salina* and *D. tertiolecta* is reported. *D. salina* is a "coper", whose cytoplasmic salt concentration is at least as high as the ambient medium. *D. tertiolecta* is an "avoider" and actively keeps salt out of the cytoplasm.

D. salina is an inhabitant of ultra-haline, neutral-alkaline waters (104, 171). Blooms in such places as the Krymsk Oblast occur in summer, which coincides with a decrease in Ca^{2+} and an increase in Mg^{2+} and SO_4^{2-} (103). Increasing NaCl concentration is increased from 2 to 4 M; from 4 to 5 M it decreases (119). Increasing NaCl results in disturbance of nucleic acid and protein metabolism. RNA per cell decreases and protein accumulates

in large amounts (122). Marre and Servattaz (102) reported high conductivity and osmotic pressure of the cell sap of *D. salina* grown in 3.9 M NaCl in comparison to those grown in 1.5 M NaCl. Based on ^{24}Na experiments, they also concluded that the cell membrane is freely permeable to sodium.

Trezzi (161) looked at the ultrastructural changes in *D. salina* following large increases and decreases in osmolarity of the culture medium.

> "When the tonicity of the nutrient medium is suddenly lowered the whole cell and single cytoplasmic structure appear equally swollen and the state of dispersion of the stroma of cellular organelles is increased. A conspicuous amount of water penetrates in every part of the cell. The mitochondria show a uniform type of swelling, while in the inner space of the nuclear envelope wide sacs appear upon hypotonic shock. The outer layer of the double membrance of the nucleus may be more permeable to water than the inner one. In chloroplasts the flattened sacs formed by paired lamellae having blind ends in the peristromium swell up giving rise to conspicuous vesicles. The permeability of water of the lamellae is greater than that of the external plastid boundary. Sudden and strong hypertonic stress causes great loss of water from every part of the cell. Shrinking can be seen in all cytoplasmic organelles together with great electron density of

their stroma. The two layers of the nuclear membrane and the lamellar pairs forming the succuli of the chloroplasts stick closely together causing a nearly total disappearance of the inner space. The formation of large vesicles after sudden hypotonic stress is due to a localized osmotic gradient. In both cases the conspicuous structural variations are reversible and the alga returns to the normal state in a short time. During the short time span necessary for the reestablishment of a new osmotic equilibrium and of a normal state of the plasmic structures, the cells suffer from a lose of motility and from an altered metabolism. A specific resistance of plasma proteins to denaturation may be involved." Concommitant with high internal salt concentration is the depressed freezing point of the cytoplasm. The organism remains motile at temperatures as low as -15°C (102). Low temperatures within the range -15°C to +11°C depress the growth of *D. salina* and in the course of eight days cause death of the cells. Some cells tolerated a temporary fall of the temperature to -30°C (170).

D. tertiolecta is a euryhaline organism growing in salinities ranging from 3.75 to 150% (75, 114). The first detailed study of the culture of *D. tertiolecta* was reported by MacLachlan (114). He established its comparatively high sodium requirement as 10 mM. Calcium and magnesium are inhibitory at high concentrations, but the inhibition can be prevented if the Ca^{2+}/Mg^{2+} ratio was maintained

at 4. Jokela (75) showed that the internal concentration of both Na^+ and K^+ increase as the salinity of the medium increases. Nevertheless the internal Na concentration stayed considerably lower than that in the culture medium at high salinities. For example, when the NaCl concentration of the medium is 15%, the intracellular concentration is 4.35%. The cell membrane is therefore not freely permeable to sodium. The cell membrane is characterized by a high phosphatidic acid content and high concentrations of C-16 saturated fatty acids. It is also characterized by its divalent ion dependent stability, its relatively high hydrophobic amino acid content and the presence of a Na-K activated ATP-ase.

Both the soluble enzymes (as exemplified by glucose 6-phosphate dehydrogenase) and particulate enzymes (pyrophosphatase and ATP-ase) of *D. tertiolecta* were found to be severely inhibited by high Na and K concentrations (above 6%) (75).

Increasing the concentration of osmotic substances causes a decline in photosynthetic oxygen evolution, then oxygen consumption, accompanied by CO_2 evolution. Both show distinct maxima at 2.8 M NaCl. Fermentation probably occurs at higher osmotic pressures as indicated by respiratory quotients (165). Glycerol production is considered to be a protective mechanism for osmoregulation in *D. tertiolecta*. With increasing concentrations of NaCl, photosynthetic incorporation of ^{14}C declines, but the percentage incorporated into glycerol increases to a distinct maximum at 2.8 M NaCl (31, 47, 165).

When transferred from a medium of 15% NaCl to 4% NaCl, 15% of the total cell glycerol is released instantaneously from the cells (75).

The upper temperature limit for growth of *D. tertiolecta* increases with NaCl concentration. Greatest growth rates occur at 0.5 to 0.75 M NaCl at 33°C (46).

REVIEW OF THE LITERATURE ON HALOPHILISM

The term halophyte literally means salt plant, and is usually used for plants that grow in the presence of high concentrations of sodium salts. A halophyte can be halophilic, salt resistant, halotolerant or haloavoidant. There is confusion in the literature as to the proper usages of these terms. According to Levitt (97) the following explanation would probably be proper. A halophilic plant is one which grows best at high salinities. On the other hand, a salt resistant plant can withstand high salinities, but grows best at lower salinities. There are two major mechanisms utilized by halotolerance and haloavoidance. The first, halotolerance, is the same as ion accumulation. The second, haloavoidance, can be accomplished in any of three ways; salt can be excluded passively, extruded actively, or can be diluted.

The salinity extremes that organisms can survive in or thrive in are very varied. Some examples are given. Certain bacteria, such as *Halobacterium salinarium* can grow in saturated NaCl (29). Selected strains of *Penicillium notatum* grow on saturated calcium acetate (143). The fungus *Scrophulariopsis parvula* grows in media with saturated NaCl. The yeast *Debaryomyces hansenii* shows respiratory activity in 24% NaCl, which is 10% of normal.

Species of algae have been reported from brine lakes, salterns, salt springs and pools with saline contents 2 to 17 times that found

in the ocean. The algae are largely Chlorophyceae, although there occur species of Myxophyceae, Bacillariophyceae and Euglenophyceae. *Dunaliella*, reported from numerous brine waters, is probably the most common of the green algae. *Chlamydomonas* has been found in the saline lakes of the Crimea. Both *Dunaliella* and *Stephanoptera* form light green areas on solid salt crusts (157). Elazari-Volcani (44) examined mud sampels taken from the Dead Sea bed. These contained green and blue green algae and diatoms. Most of the green forms resembled *Dunaliella*, with lesser amounts of *Scenedesmus bijugatus*, *Pediastrum simplex* and *Ulothrix*. The blue greens resembled *Aphanocapsa littoralis*, *Aphanothece halophytica* or an *Oscillatoria*. Of the diatoms, forms of *Melosira*, *Navicula*, *Pinnularia*, *Gomphonema*, *Cymbella* and *Synedra* occurred. Each liter of Dead Sea water contains approximately: 143 g. magnesium chloride, 87 g. sodium chloride, 37 g. calcium chloride, 11.5 g. potassium chloride and 5 g. magnesium bromide.

Species of *Enteromorpha* occur in the Great Salt Lake as well as from fresh waters. Fluctuating amounts of salinity produce specimens that have characteristics of several species in a single frond (157). The blue green algae *Sprirulina subsala*, *Phormidium tenue* and others grow and multiply in 3 M NaCl (65, 84).

A few examples of halophilism of higher plants are given. Seedlings of *Atriplex vesicaria* were established in 1 M NaCl (16). At

100-200 mEq/liter of Na^+, K^+ or Mg^{2+} Cl *Atriplex nummularia* grows optimally. There is still good growth at 300 mEq/liter (57). *Halogeton glomeratus* survives at 1.4 molal NaCl (137). The optimal level of NaCl for growth of the mangrove *Avicennia marina* is 1.5%, one half the concentration of sea water (30). Succulent halophytes such as *Sueda depressa, Salicornia europaea* and *Spergulia marina* germinate and develop in 5% NaCl (162). Leaves of *Nitraria schoberi* (the most tolerant among woody plants) may contain 14% of their dry matter in the form of NaCl, 57% as total salts (158).

Mechanisms of Osmotic Regulation

The following are mechanisms of osmotic regulation utilized by halophilic plants and microorganisms:

A. Development of cell membranes highly permeable to ions.

A membrane which is highly permeable to sodium enables the cell to adjust rapidly to high osmotic pressure changes. This requires adaptation of a cell's enzyme system, nucleic acids, ribosomes, etc., to function at high as well as low ionic concentrations. This mechanism is suggested as being responsible for the osmotic regulation of *D. salina* (102). Another species of the halophilic organism, *D. parva,* is freely permeable to sucrose, inulin, starch and even polyvinylpyrollidone with a molecular weight of 20,000 to 40,000 (53). It is impermeable however to dextran (mol. wt. 80,000). The

mycelia of a salt tolerant strain of *Penicillium notatum* accumulate high intracellular concentrations of copper when grown on metallic copper surfaces (154). Kylin and Gee (88) showed that a halophilic mangrove (*Avicennia nitrida*) possesses a Na extrusion pump, but it is inactivated at high concentrations of NaCl (0.2 to 0.4 M). Similar inactivation also occurs with *Atriplex subcordata, Suaeda maratima* and *Astes tripolium* (168).

B. Avoidance, including active Na extrusion.

Many plant halophiles, including *Spartina townsendii, Limonium latifolium* (3) and the mangroves (6) posses salt glands which extrude NaCl against a concentration gradient (144). In the case of *Tamarix aphylla,* the glands show no apparent selectivity between Na^+ and K^+ ions (14). Even Rb was taken up from the nutrient solution and secreted by the glands (160). Single celled organisms may use vacuoles to maintain a steady state low concentration of NaCl. The non-photosynthetic flagellate *Choanogaster plattneri* is found in water of 20% salinity. The cells have a complex vacuolar system, producing smaller accessory vacuoles that empty outside the cells and that decrease in activity when the salinity of the medium is lowered. The vacuoles are said by Pochman (136) to contain a higher concentration of salt than the medium and serve to excrete salt. The contractile vacuoles of *Chlamydomonas moewusii* have an entirely different function, the elimination of water. A mutant strain lacking contrac-

tile vacuoles survives and grows only if the osmotic pressure of the medium exceeds 1.5 atm (59).

For algae, sodium pumps have been described since Scott and Hayward (146) described a sodium excretion mechanism in *Ulva lactuca*. The mechanism of this active transport was envisioned as involving a cation sensitive ATP-ase system as demonstrated for erythrocytes (36, 137). As far as completing the comparison, Eppley in 1962 (45) stated that "an experimental approach might be facilitated if an algal equivalent of the erythrocyte ghost were available." Jokela, in 1969 (75) isolated naturally wall free, ghost forming membranes of *D. tertiolecta* and detected Na-K ATP-ase activity.

C. Development of an active exchange-transport system.

Halobacteria avoid excessively high sodium content of the cell by an active inward transport of potassium. K^+ concentration is much higher than in the medium, and in some cases the value found intracellularly was close to the limit of solubility for KCl (29). Adaptation of the cell's enzyme structure is necessary for functioning at high ionic strength. Many of the enzymes are reversibly inactivated in the absence of high concentrations of neutral salts (91).

D. Utilization of inert neutral compounds as osmotic agents.

The maximum proline concentration that *E. coli* can concentrate internally has been shown to be dependent on the osmolarity of the suspension medium; the labeled amino acid pool in *E. coli* has been shown to be proportionally released when the cells are washed with

low osmotic strength solutions (20). Oxalate synthesis in *Atriplex* spp. has been shown to be related to the external osmotic pressure (132). Polyalcohol production of yeast is influenced by the presence of high salt concentrations (131). Glycerol production by *D. tertiolecta* in response to increasing osmotic pressures has been mentioned.

E. Salt dilution.

Rhizophora mucronata does not excrete salt, yet its growing leaves retain a constant concentration (510 to 560 mEq/liter) though receiving 17 mEq Cl/day (6). During growth, sufficient water is absorbed to prevent increase in concentration. This dilution of cell sap during growth has been found in some moderately salt tolerant halophytes (97). Many other plants avoid increase in concentration by increasing succulence. Cells, especially parenchyma cells enlarge, avoiding excessive salt concentrations (139).

High Salinities and Cellular Adaptations

The adaptations of some cellular constituents to high salt concentrations are related.

A. Cell envelopes.

Halobacteria do not have clearly separable wall and membrane structures (142). The term cell envelope is used to designate structures enveloping the cytoplasm. The cell envelope requires a high salt concentration for its maintenance; at low concentrations it loses

its rigidity and collapses. Chemicals which do not possess a strong net charge in aqueous solutions do not protect the structure of the envelope (123). The hypothesis of Abram and Gibbons (1) is that the cell envelopes of the halobacteria "are held together rather loosely by hydrogen bonds, Coulomb forces or 'salt' linkages, and in the presence of high concentrations of NaCl the electrostatic forces are screened so that the bonds hold the organism together in a rod shape." The cell envelopes of the halobacteria have very few or no higher fatty acid residues, and in this way differ pronouncedly from other bacteria (27, 91). The bulk of the lipid is an analogue of diphosphatidyl glycerophophate (147). The proteins of the cell envelope of halobacteria are very acidic (21).

It is of interest that in the cell membrane of *D. tertiolecta* long chain fatty acids are absent, at least when the organism is cultured at high salinities. There is also a high phosphatidyl glycerol content and a predominantly acidic amino acid composition (75).

B. Nucleic acids.

According to Kessler (83) high osmotic pressures induce changes in copolyribonucleotides synthesized from equimolar concentrations of ADP, GDP, CDP and UDP. He suggests that osmotic stress is coded into a changed composition of polyribonucleotides which may lead to adaptive reactions by serving as primers for RNA dependent RNA polymerase. Siegel (152) has demonstrated the role of nucleotides, especially IMP, in the recovery of *Penicillium* from extreme salt effects.

C. Ribosomes.

The ribosomes of *H. cutirubrum* are stable only in solutions containing KCl close to saturation (4M) and Mg^{2+} in the concentration range 0.1-0.4 M. The KCl requirement is quite specific, and cannot be replaced by iso-osmolar NaCl (12). With regard to higher plants, it has been shown that the ribosomes of salt sensitive citrus species are less stable than those of more resistant species, but were able to acquire an increase in stability (83).

D. Proteins and enzymes.

In a culture of callus tissues from cabbage leaves, it was found that salts slow down amination and amidation, as well as the transfer of sulfides to S-containing amino acids (148). Shevyokova (148) suggests that an increase in derivatives of S-amino acids may be one reason for the unfavorable effect of salts.

Baxter and Gibbons (11) made the important discovery that the enzymes of extremely halophilic bacteria are adapted to function at the very high salt concentrations found within the cells. Since then about twenty different enzymes have been reported, and all data fit the general contention that the enzymes of extremely halophilic bacteria are extremely halotolerant, and in most cases even strikingly halophilic (11, 29, 91). These enzymes are irreversibly inactivated in the absence of high concentrations of salts. For example, *Halobacterium salinarum* excretes a protease which is possible to chro-

motograph only in the presence of 3.4 M NaCl (127). Another specific property of their proteins was the absence of half-cysteine residues, in opposition to the non-halophilic *E. coli* which has 0.6 moles half-cysteine per 100 moles of amino acid. Stroganov (158) suggests that the bacteria adapt to high salinity by binding of the mineral element (158). Haloavoidant organisms and non-halophiles usually have enzymes that are salt sensitive, as is the case with *D. tertiolecta* (75).

The capabilities of some enzymes under stress are enormous. *In vitro* horseradish peroxidase activity is present in 10 M lithium chloride. Considering the coordination number of the ions, a 10 M LiCl solution can have little or no uncoordinated water (153).

HALOTOLERANCE - EXPERIMENTAL INVESTIGATIONS

Growth of *D. salina* was reported by Marre (102) to occur in saturated NaCl solutions. McLachlan (114) demonstrated *D. tertiolecta* growth in 2.5 M NaCl, the highest concentration tested. This investigation corroborates these results. It was also found that *D. tertiolecta* growth occurs in saturated NaCl, though greatly attenuated ($^1/_4$ the rate of growth in 2.5 M NaCl).

All cultures were grown in Hutner's Marine *Chlamydomonas* medium, with variation only in the major chloride (69) (See Table 1). The pH was adjusted to 7.2 with KOH. This glass electrode measurement proved to be optimum for increase in cell number for *D. salina* cells grown in 300 g/l NaCl (See Table 2). It must be kept in mind that such pH measurements are inaccurate, due to dilution of water by the concentrated salts. The light intensity of all experiments was a continuous 3000 lux.

At room temperature the effect of various electrolytes and glycerol on *Dunaliella* growth are shown in Table 3. NaCl, KCl, $MgCl_2$, $CaCl_2$ and glycerol were added to the medium in concentrations yielding an osmolarity equivalent to $55^0/_{00}$ NaCl.

Table 1. Hutner's marine chlamydomonas medium (69), pH adjusted to 7.2 with KOH.

Metal chloride	Xg/liter
EDTA	0.50 g
$K_2HPO_4 \cdot 3H_2O$	0.26 g
$MgSO_4$	1.22 g
Glycine	2.50 g
K-acetate	2.00 g
H_3BO_3	244.00 mg
$ZnCl_2$	62.00 mg
$Ca(NO_3)_2 \cdot {}_4H_2O$	147.00 mg
$MnSO_4 \cdot H_2O$	31.00 mg
$Na_2MoO_4 \cdot {}_2H_2O$	25.00 mg
$FeCl_3 \cdot 6H_2O$	14.00 mg
$CoCl_2 \cdot 6H_2O$	1.00 mg

Table 2. Effect of initial pH of Hutner's marine chlamydomonas medium on growth of *D. salina*. Cultures initially contain 250 ml of 5 X 10^4 cells/ml.

Glass electrode pH	% increase in cell number after 3 weeks
5.0	all dead
6.0	300
6.8	1000
7.7	1300

Table 3. Change in cell number of 250 ml cultures in Hutner's supplemented with chlorides and glycerol of osmolarities comparable to $55^{0}/_{00}$ NaCl. Incubation period is three weeks. The initial cell concentration (X) is 5×10^{4} cells/ml.

	D. salina	*D. tertiolecta*
$NaCl_2$	14X	31X
$MgCl_2$	0X	5X
KCl	0X	0X
$CoCl_2$	0X	0X
Glycerol	0X	1X

The highest growth rates occur in NaCl. Slower division in $MgCl_2$ and glycerol was evident. All measurements were done with visible microscopy and a Levy hemocytometer. Lowering the temperature extends survival time for $CaCl_2$ cultures. (Figure 1). This fact will be elaborated upon in the next section.

To ascertain if diffusion of the major cation through the plamsa membrane is necessary for osmoregulation, survival of *D. salina* in $choline^+$ $chloride^-$ was determined. One ml aliquots of a late log culture of *D. salina* grown in 150 g/l NaCl Hutner's were transferred to either of two solutions. The first solution contained two ml of the identical medium. The second contained two ml of 35 g/l NaCl Hutner's supplemented with 27.6 g $choline^+$ $chloride^-$ / 100 ml to make it isotonic. After 10 minutes, there was a 38% drop in viability in the choline chloride solution.

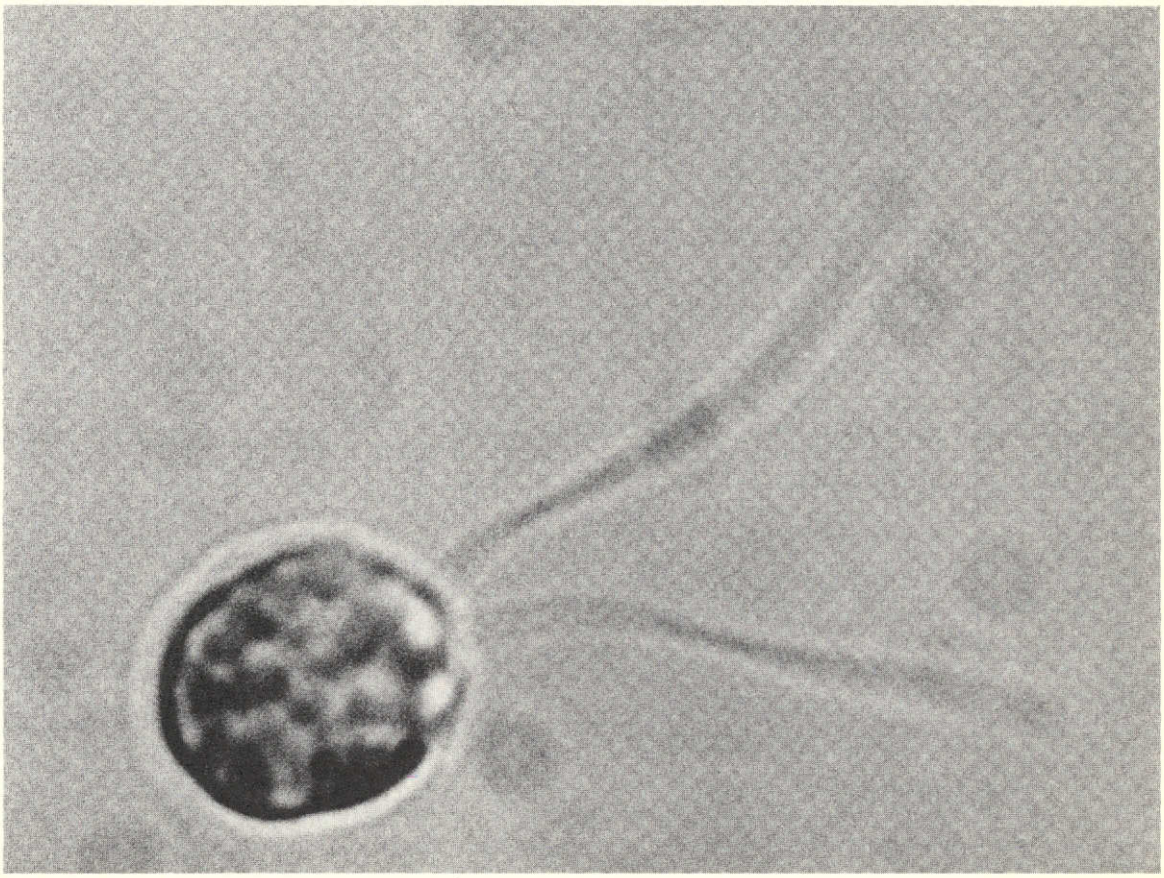


Figure 1. *Dunaliella salina* in saturated $CaCl_2$.

Optical resolution. Cell diameter lengthwise is 10 μm.

Osmotic Regulation

The conclusion of Marre (102) that *Dunaliella salina* plasma membranes are freely permeable to sodium is based almost entirely on his ^{24}Na experiments. Our studies have shown that his interpretations of the data were almost completely fallacious.

Marre suspended aliquots of algae in the original media indicated below and dialyzed them for ten hours against 20 volumes of the final medium containing $^{24}NaHCO_3$.

NaCl in medium		Na^{24}CPM / cc	
original	final	medium	cell sap
2.50 M	7 M	165,000	81,000
1.25 M	7 M	163,000	112,000

The original medium did not contain the isotope. This was introduced with a specific activity as high as practical without altering the Na^+ ion concentration of the medium. Before monitoring radioactivity of the algae, Marre centrifuged the cells for 20,000 g for 20 minutes and decanted the medium. He somehow assumed that the intercellular volume of the packed cells was less than 3% of the total volume. No basis for this assumption is given.

A follow-up experiment involved exclusive use of iso-osmotic solutions.

NaCl conc. of the medium	Na^{24} cpm / cc after 10 hours in the medium containing the isotope		30 minutes after transport of cells from med. containing Na^{24} to isotope free med.
	In medium	In cell	In cell
2.50 M	129,000	71,000	360
3.75 M	129,000	94,000	150

On the basis of these two experiments, along with the observations of Trezzi (161) that the cells shrink in hypertonic solutions and then regain their original shape—that all the subsequent literature on the passive Na^{+} intracellular accumulation is based.

Assuming Marre's interpretation of his data was correct, we proceeded to look for halophilic or halotolerant enzymes in *D. salina*. This search seemed to be called for since there has never been reported a halophilic or halotolerant enzyme isolated from an alga. Both glucose-6-phosphate dehydrogenase and catalase had attenuated activities with increasing NaCl concentrations. Increasing NaCl concentration from $6^{0}/_{00}$ to 100 $^{0}/_{00}$ resulted in a 90% decrease in glucose-6-phosphate dehydrogenase activity. At $250^{0}/_{00}$ NaCl there was another tenfold loss in activity.

To make precise estimates of osmotically active substances in *D. salina,* the following determinations for cell volume, sodium, potassium and glycerol were performed.

Determination of Intracellular Volume of *D. salina* Cells Using a Bluedextran Indicator Method

Cells were harvested at the end of their log phase of growth at 25° in $150^{0}/_{00}$ NaCl Hutner's and resuspended in identical sterile solution. Two hematocrit tubes were filled with aliquots of the suspension and centrifuged at 2,500 g for 10 minutes. The ratio of packed cell volume to total volume of liquid was then measured.

Four ml aliquots of the algal suspension were centrifuged under the same conditions as the hematocrit tubes. The supernatants were decanted, the sides of the centrifuge tubes carefully dried, and then the packed cells were resuspended in a Blue Dextran solution of known concentration and Optical Density at 580 mm. The cells were then recentrifuged under the previous conditions (2,500 g for 10 minutes). The supernatant was decanted, millipore filtered, and then the Optical Density determined. By comparing standard Blue Dextran dilution plots to the decrease in O. D. of the dye solution caused by dilution of the dye by intercellular space liquid, the volume of the intercellular space was determined. The intercellular volume was 32% of the packed volume, ten times Marre's figure.

Assay of Glycerol Using Glycerol Dehydrogenase

D. salina cultures in late log phase were centrifuged at 2,500 for 10 minutes, and then the supernatant analyzed for glycerol.

1. To each tube containing from 0 to .4 μ moles of standard glycerol solution, or similar amount of sample, or both, the following solutions were added:
2. 1.9 ml of Sorensen's glycine II buffer, pH 9.5
3. 0.2 ml of glycerol dehydrogenase (Worthington, from Enterobacter aerogenes) (2mg/3 ml), prepared immediately before use.
4. Distilled water to bring volume up to 3 ml.
5. 0.1 ml of DPN solution (6.6 mg/ml)

Optical density at 340 mu with appropriate standards recorded for twenty minutes.

To determine intracellular glycerol concentrations, packed cells were washed twice with 100 volumes of fresh medium at 4°, then freeze-thawed and centrifuged. The supernatant was analyzed in the manner indicated above. Results are shown in Table 4.

Table 4. Intra and extracellular glycerol, Na and K in late log *D. salina* culture grown in 150 g/l NaCl Hutner's medium. Values are means of three determinations.

	Intracellular		Extracellular	
Compound	Molarity	Osmolarity[a]	Molarity	Osmolarity[a]
Glycerol	1.640 ± 0.260	1.64	0.004	0.004
Na	0.534 ± 0.237	1.07	2.560	5.120
K	0.084 ± 0.034	0.16	0.044	0.088
		2.87		5.212

[a] Osmolarities include anion and assume (without justification) a 1 : 1 anion-cation ratio, in a completely free liquid cell.

Determination of Intracellular Sodium and Potassium Content

Triplicate samples of packed cells were digested in 2 ml of 17:3 nitric-perchloric acid and heated until solutions were clear. Solutions were then diluted with deionized water and analyzed with flame atomic absorption apparatus. The absorbancy of sodium at 589 nm was measured and the concentration determined by interpolating in the standard curve of Na. Potassium absorbance was measured at 768 nm. Results are summarized in Table 4.

Apparently glycerol is the principal osmotically active intracellular substance in *D. salina*. Sodium ion extrusion and potassium ion accumulation is occurring. In light of these results investigations into glycerol-salt interaction with *D. salina* enzyme are planned for the near future.

The effect of salinity on mercury toxicity was also investigated. Mid log cultures of both Dunaliella species were grown at $35^0/_{00}$ or $150^0/_{00}$ NaCl and exposed to various concentrations of $HgCl_2$ for two hours. The per cent of motile cells before and after treatment was counted using light microscopy and a hemocytometer. Results are plotted in Figure 3. Interesting to note is that Hg tolerance as reflected by ID_{50}'s is roughly doubled at higher concentrations of NaCl.

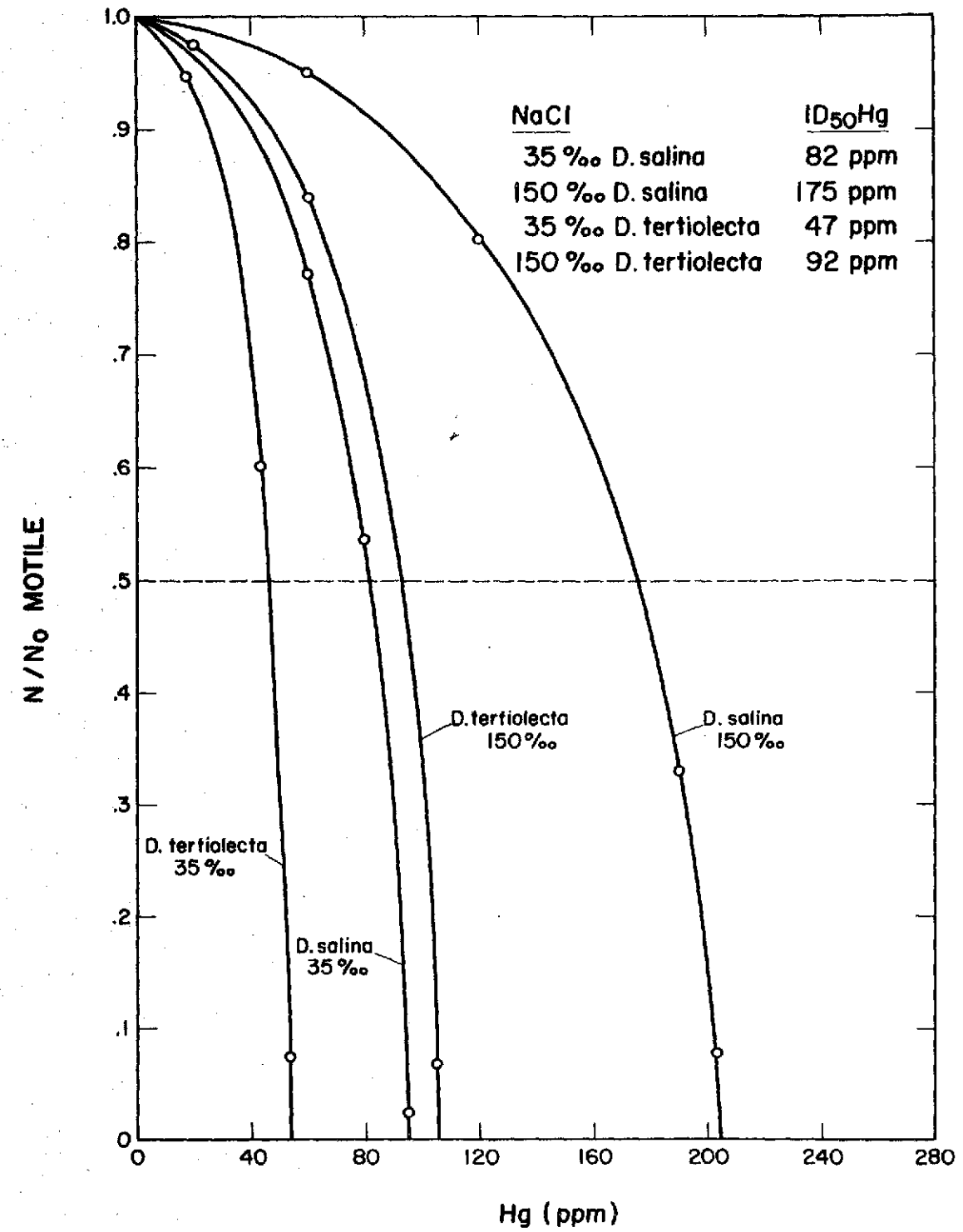


Figure 3. Response of *Dunaliella* to $HgCl_2$ in different concentrations of NaCl. Each determination represents a count of the number of motile cells per 1000 cells before treatment and after two hours of incubation with prescribed mercury levels.

REVIEW OF THE LITERATURE OF CRYOTOLERANCE

There is an apparent contradiction between the use of very low temperatures to store cells and tissues for long periods of time and the finding that living cells are usually destroyed by sub-zero temperatures (48). With the exception of nuclear processes and combining of free radicals (140), the rates of all physical, chemical, and therefore biological processes are temperature dependent (110). When processes depend solely on the mean velocity of molecular motion, the decrease in rate with decrease in temperature is proportional to the fractional change in absolute temperature, and is therefore small over ten or twenty degree intervals. Diffusion is such a process. But for reactions requiring collision of molecules having energies over some threshold value, the effect of temperature on reaction rate can be tremendous. Thousands of enzymatically catalyzed reactions are occurring in living cells and most occur in the correct temporal and spatial sequence to be effective. Since the temperature coefficients (Q_{10}'s) and activation energies of these reactions are not identical, a decrease in temperature is likely to upset the balance (74).

Lowering the body temperature 10°C or 20°C is lethal to most homoiothermic animals and profoundly alters the metabolic rates of all poikilothermic organisms. These alterations are reversible in most poikilotherms as long as no water phase change is involved. Examples

of organisms that can withstand low temperature is given.

Bacteria

Psychrophiles are bacteria capable of growth at 0°C, but subject to enhanced rates at higher temperatures (153). The implication that there is an actual preference at or near 0°C is incorrect (72). It has been suggested that they should be called psychrotrophic, and that the term psychrophilic be reserved for organisms that grow optimally below 30°C (43, 156). Psychrophiles are ubiquitous, found not only in soils and oceans, but also in dairy products, meats and fruits.

Mostly, these bacteria are gram-negative, non-spore forming rods, including the following genera: *Serratia* (5, 34, 156), *Flavobacterium, Archromobacter, Pseudomonas* (156), *Salmonella, Proteus, Bacillus* (52), and *Lactobacillus*, Cocci, namely *Streptococcus* (32), *Micrococcus* (52, 117), and *Rhodococcus* have been reported. Strictly anaerobic psychrophiles seem to be rare in most habitats, although sulfate reducers occur in ocean deposits (159) and some spore forming anaerobes such as *Clostridium* will grow well at 6°C (13). Bacteriophage with growth temperature maxima only slightly above that of the psychrophilic host have been reported (130). ZoBell (173) has been able to culture 76 out of 88 kinds of marine bacteria at sub-zero temperatures. This is significant since over 80% of the ocean floor is 1700 m or more deep with temperatures $\leq$ 3°C (7).

Protozoa

Wolfson (165) was able to supercool suspensions of *Paramecium* to -14°C without completely stopping ciliary action. Viable cells of *Paramecium* recovered and multiplied after exposure to liquid nitrogen temperatures (155). Some amoebae survive supercooling to -5°C. (25). There do not seem to be any reports of growth or multiplication of protozoa in sub-zero temperatures.

Fungi

There exists a *Penicillium* mutant capable of growth in ordinary broth or solid media saturated with acetates or chlorides of Na, K, Mg, Ca or Sr that will grow at constant 4°C or with 16 hours per 24 hours at -30°C just as well as at constant 25°C (150). Vital activity of this same organism was found in liquid ammonia-glycerol media at -40°C (49). Cells of the yeast *Saccharomyces cerevisiae* survive in prolonged suspension supercooled to -16°C (108), and are capable of surviving liquid N_2 temperatures if slow cooled and fast warmed (70, 100, 111). Isolated mitochondria of the same organism can be preserved intact at -190°C without addition of cryoprotective agents (8). The freeze-thaw response of *Neurospora crassa* is very similar to *S. cerevisiae* (9). The list of psychrophilic fungi include *Candida, Rhodotorula, Aureobasidium, Geotrichum* and *Phoma* (161). Lichens such as *Cladonia, Lobaria,* and *Umbilicaria* are capable of withstanding temperatures well below 0°C (66).

Algae

The plants of perpetual snow and ice are often related to planktonic algae of lakes and streams (157). The vegetation is largely made up of algae, although moss protonema, fungi and bacteria also occur. The algal forms generally grow close to the surface. Many forms multiply and grow in the melted snow and are therefore living at temperatures no lower than the freezing point. Oxygen is always ample.

Cryophytes are frequently classified on the basis of color. The green color of the snow of European and Arctic regions is associated usually with limestone. Species of *Chlamydomonas*, *Ankistrodesmus* and *Mestaenium* are common (157). *Euglena* also occur (82). Red snow is common all over the world. The algae responsible for the color are species of *Chlamydomonas*, *Scotiella* (28), *Chionaster*, *Raphidonema* *Gleocapsa* and some diatoms (90). Yellow or yellow-green snow is caused by *Protoderma*, *Scotiella* and *Chlorosphaera* (98, 157).

Active life for these forms poses two problems, the ability to survive freezing during extremely cold nights and the capacity to carry on metabolic reactions required for growth and assimilation when the day temperature is in the neighborhood of 0°C. The first problem is common to many plants and is usually defined as that of frost resistance. One mechanism of defense against low temperatures can be a high osmotic pressure of the cell sap, correlated with a lowering

of the freezing point. This is the case with *Dunaliella salina*. However, high internal osmotic values do not always result in cold tolerance; for example in *Griffithsia*, an intertidal red alga, the higher osmotic pressure of the apical cells is associated with a higher sensitivity to cold damage (15). Drying of intertidal algae is sometimes related to freeze tolerance. For example, respiration in *Fucus* has been measured at -15°C. *Fucus* is a prominent Arctic intertidal alga (77).

Just as an aside, at least 23 strains of green algae (*Chlorella, Ankistrodesmus, Coccomyxa, Scenedesmus, Euglena*) and blue green algae have been frozen in the laboratory to liquid nitrogen temperatures and remained viable (23, 67, 70).

Higher Plants

Aside from conifers, winter evergreen species include *Pteridophytes*, and the angiosperms *Asplenium, Dryopteris, Polypodium, Rhododendron, Arctostaphylos* and *Lauris* (153). These forms maintain some degree of metabolic activity at sub-zero temperatures.

Higher Invertebrates

Survival of intertidal mollusks of temperatures down to -5°C have been recorded (74). Sea urchin eggs can survive in the -5°C to -15°C range (4). The intrinsic nontoxicity of glycerol as a cryoprotectant is supported by many observations of high concentrations of this compound found in insects. The most dramatic example is of an Alaskan

insect which elaborates glycerol to a whole body concentration of 2.5 M and is uninjured by months of freezing at -40°C, at which temperature concentrations in excess of 6 M will be produced (10, 128).

Vertebrates

In fish and turtles, core temperatures of -1°C have been found (35, 144). Human erythrocytes are routinely stored at liquid nitrogen temperatures. Bare skinned mammals such as swine tolerate -50°C (153). Recently mouse embryos were frozen to -269°C. When reimplanted after thawing they developed into normal mice (165).

Effects of Cold and Frost on Cells

The effects of cold and frost on different cell organelles including membranes (33, 49, 62, 87, 96, 109, 112, 116, 125, 135), chromosomes (95, 96, 98, 126), chloroplasts (60, 61, 62), mitochondria (8, 60, 87), ribosomes (73), vacuoles (61, 79) and lysosomes (135) are well documented. This is also the case with chemical constituents such as proteins (19, 23, 34, 48, 58, 60, 61, 62, 73, 78, 81, 92, 93, 95, 96, 98, 115, 126), lipoproteins (61), lipids (2, 84, 85, 87, 96), sugars (60, 61, 79, 113) and nucleic acids (84, 96, 98, 126). Rather than discussing each constituent individually, general theories of cryodamage, tolerance and avoidance will be discussed.

Plants, as poikilotherms, are unable to develop low temperature avoidance. They are either tolerant (hardy) or else they show damage. A number of theories of frost injury and tolerance were compiled by Levitt (94). They will be summarized.

A. Armchair theories

Perhaps the oldest is the "caloric theory" which proclaimed that frost resistance results from the release by the plant of enough heat to prevent freezing. This theory was disposed long ago since it imples avoidance of both frost and low temperature injury, whereas frost resistance is actually tolerance.

The second oldest is the "rupture theory". According to this theory, cellular expansion caused by ice formation resulted in cell

rupture. However, later investigations showed that frozen tissues actually contract.

B. Frost precipitation theory

Gorke (54) in 1906 found that freezing plant juice precipitated the proteins and that this precipitation was prevented by adding sugar. He therefore explained frost injury as a precipitation of the protoplasmic proteins caused by the concentration of the cell salts that occurs on freezing, and frost resistance as a result of the protection of the proteins by the sugars that accumulate on hardening. Gorke's protective effect by sugars was corroborated using grains but not in most observations. Furthermore, there are many plants that cannot become resistant in spite of high sugar content, so his explanation of frost resistance is inadequate. Finally, it has been possible to increase frost resistance by inducing the uptake of salts (141).

C. Iljin's mechanical stress theory (71)

Iljin was struck by the pronounced cell collapse that occurs on extracellular ice formation and concluded that the protoplasm of such cells must be subjected to stresses and strains. He determined that injury occurred only during the thawing, mainly because of his success in preventing injury by simply thawing in strongly plasmolyzing solutions. The factors associated with hardiness can be explained logically by Iljin's theory. The smaller the cell size, the greater the specific surface and therefore, the less the strain developed due

to any one degree of cell contraction. The greater the sugar concentration, the smaller the water loss and therefore the less the cell contraction and the stress at any one freezing temperature. An increase in bound water produced by penetrating cryoprotective agents would have the same effect. However, the theory gave no explanation for the greater injury caused by intracellular than by extracellular freezing, nor for the greater resistance of hardy protoplasm to stresses and strains. Nor could it explain the injury to animal cells without stiff cellulose walls.

D. Intracellular freezing theory

Intracellular freezing is consistently fatal at temperatures that fail to injure when freezing is extracellular. Thus, rapid cooling to -30°C results in 99.9% killing of yeast cells; slow cooling to the same temperature results in 50% kill (108). Such evidence has frequently led to the suggestion that all frost resistance is due to the avoidance of intracellular freezing. Scarth (143) was the first to suggest that the higher permeability of hardy cells to water would favor this avoidance, and to later produce evidence that hardy cells do actually show an avoidance of intracellular freezing. For example, sea urchin eggs are more resistant to rapid freezing injury when fertilized than when unfertilized, and this is correlated with four times greater membrane permeability when fertilized. Chambers and Hale (25) first showed that cell membranes are essentially impermeable barriers to ice seedings, except at temperatures below -40°C.

E. The sulfhydryl-disulfide theory

According to this theory of Levitt's (94) frost injury is the result of unfolding and therefore the denaturation of the protoplasmic proteins. This results from the mutual approach of the protein molecules (due to their dehydration during freezing) until they are close enough for the formation of intermolecular SS bonds. Levitt admits "although direct evidence in its favor is admittedly still lacking, there is much to recommend it as a working hypothesis." The hypothesis is supported by the following facts: 1) increase in protein SS is found when freezing does not result in injury; 2) the attenuated effect of freezing if done under low O_2 tensions (150); 3) general apparent increases in protein SH on hardening; 4) greater injury during intracellular freezing may result from the compression of the dehydrated molecules between expanding ice loci. This would bring SH and SS groups closer together; 5) the protective effect of glycerol in the case of animal cells can be explainable by the binding of the glycerol molecules (by hydrogen bridges) to the SH groups of the protein molecules (replacing removed water molecules), thus keeping them too far apart for interaction; 6) the increase in GSH oxidizing ability of the cell that occurs during hardening, which would tend to eliminate GSH — a substance capable of triggering SH-SS interchange reactions in protein; 7) enzymes are frequently prepared from tissues killed by freezing and since their activity is retained, unlike other

proteins in the dead protoplasm, they cannot have been denatured. But the enzymes so prepared are soluble ones which would not be held in the protoplasmic framework, and therefore would remain too far apart for SH-SS interchange.

CRYOTOLERANCE - EXPERIMENTAL INVESTIGATIONS

The premise of necessary Antarctic Dry Valley sterility as promulgated by Horowitz and Cameron in 1972 (68) is contradicted by the motility capabilities of *D. salina* at -15°C. Studies reported by this author concerning uptake of radioactively labelled organic compounds by "greenhouse sludge" in a Don Juan Pond simulator were reported in University of Hawaii Botanical Science Paper Number 31. It is a fact that there is metabolism of autotrophic eukaryotes at sub-zero temperatures. The high internal osmolarity and concommitant extensive freezing point depression capabilities (3 M glycerol depresses freezing point to -9°) were another reason for it being a likely candidate for being a cryophile.

Azenic cultures of *D. salina* and *D. tertiolecta* were grown at room temperature in Hutner's medium supplemented with 2.5 molar sodium chloride and adjusted to pH 7.2. A constant light intensity of 3000 lux candles was maintained. At late log phase with approximately 5 X 10^7 cells/ml, aliquots of both cultures were equilibrated to 25, 6, -1 and -8 degrees respectively under identical light intensities. After two hours, each culture was innoculated with a sterile, equimolar solution containing 40 µCi/ml ^{14}C labelled sodium carbonate. After an additional 36 hours, aliquots were withdrawn from each culture, millipore filtered (.22 M) and washed. Cells plus filters were placed in scintillation cocktail. One day was allowed

for bleaching of cocktails, and then radioactivity was read using a scintillation counter. Appropriate standards were used for calculation disintegrations per minute. As indicated by Figure 2, CO_2 reduction is appreciable below zero degrees for both species. Although extrapolations to the baseline as drawn do not reach below minus ten degrees, further experimentation is necessary to determine the actual lower limit of carbon dioxide reduction.

In an attempt to isolate labeled photosynthates, produced at -6°C, high specific activity 1 mCi/ 1.6 mg $NaHCO_3$ was utilized. A few low molecular weight organic compounds have been isolated, but not presently positively identified. They are very convincing evidence that more than non-specific absorption is occurring at -6°C.

For low temperature studies, both species of *Dunaliella* provide excellent visual indicators of their osmotic state and viability. These indicators are cell shape and motility. We have observed that approximately nine times out of ten, structural integrity and motility of individual cells occur together. The value of viability determinations for low temperatures is not mitigated if observations are made after cultures are returned to room temperature, being that no phase change of the salty medium is involved. The high correlation of structure and function which can be observed using light microscopy provides an efficient, yet non-complex system for evaluating the effect of any sort of parameter on the low temperature viability

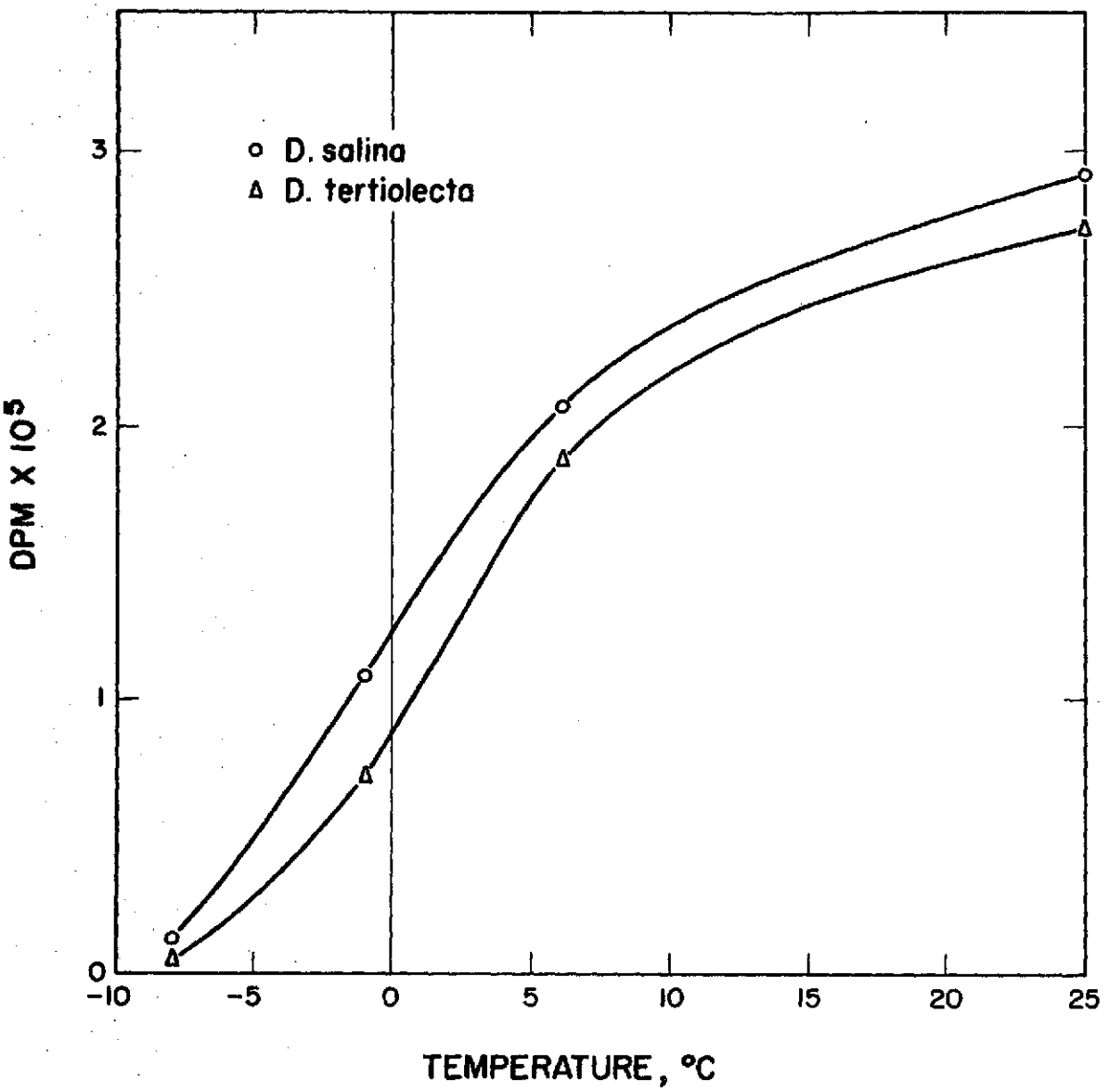


Figure 2. $^{14}CO_2$ reduction by *Dunaliella* in 150 °/₀₀ NaCl at temperatures approaching -10°C.

of *Dunaliella.*

Dunaliella cells in mid-log phase growing in 2.5 M NaCl Hutner's divided into 12 aliquots per species and each of the below treatments were performed in triplicate for three weeks:

25° constant 3000 lux illumination

25° dark

-6° constant 3000 lux illumination

-6° dark

The following table indicates cell number before and after three week treatment.

		Viable cells/.1mm^3		
		Pretreatment	Post-treatment	% change
D. salina				
	25° light	63	182.00 ± 28.00	288.8
	25° dark	63	4.00 ± 3.00	6.6
	-6° light	63	.55 ± 0.58	0.9
	-6° dark	63	26.60 ± 30.00	42.2
D. tertiolecta				
	25° light	31	63.00 ± 30.00	180.0
	25° dark	31	11.00 ± 6.00	31.4
	-6° light	31	13.00 ± 2.00	41.9
	-6° dark	31	6.50 ± 4.30	20.3

The increased low temperature survival in the dark for both species is most interesting. It was observed by R.T. Wilce (166) and other investigators that the intensity of light available to many Arctic deep water benthic marine algae is almost negligible (1-30 candle power) for most of the year, yet they managed to survive. He goes so far as to hypothesize facultative heterotrophy.

Viewing the Dunaliella low temperature survival findings, perhaps low light intensities, though they are not conducive to active metabolism (i.e., photosynthesis), do favor survival (preservation) before spring thaw.

REFERENCES

1. Abram, D. and N. E. Gibbons. 1961. The effects of chlorides of monovalent cations, urea, detergents and heat on morphology and turbidity of suspensions of red halophilic bacteria. Can. J. Microbiol. 7:741-750.

2. Adams, Bryant L., Vern McMahon and Joseph Seckbach. 1971. Fatty acids in the thermophilic alga, Cyanidium caldarium. Biochem. Biophy. Res. Commun. 42:359-365.

3. Ariszm W. H., I. J. Camphuis, H. Heikens and A. J. van Tooren. 1965. The secretion of the salt glands of Limonium latifolium. Acta Bot. Neer. 4:322-338.

4. Asahina, E. 1961. Intracellular freezing and frost resistance in egg cells of the sea urchin. Nature (London) 191:1263-1265.

5. Ashwood-Smith, Michael J. and Carol Warby. 1971. A species of Pseudomonas, a most useful bacterium for cryobiology 8:208-210.

6. Atkinson, M. R., G. P. Findlay, A. B. Hope, M. G. Pitman, H. S. W. Saddler and K. R. West. 1967. Salt regulation in the mangroves Rhizophora mucronata Lam. and Aegiolitis annulata R. Br.. Aust. J. Biol. Sce. 20:589-599.

7. Baas-Becking, L. J. M. 1930. Contributions to Marine Biology, Stanford University Press, Palo Alto.

8. Balcavage, Walter X., Jeanne C. Beck, David P. Beck, John W. Greenwalt, John H. Parker and James R. Matoon. 1970. Cryobiological studies of yeast mitochondria. Cryobiology 6:385-394.

9. Barnhart, E. R. and Claude E. Terry. 1971. Cryobiology of Neurospora crassa I. Freeze response of Neurospora crassa conidia. Cryobiology 8:323-327.

10. Baust, J. G. and L. K. Miller. 1970. Mechanisms of freezing tolerance in an Alaskan insect. Cryobiology 6:258.

11. Baxter, R. M. and N. E. Gibbons. 1954. The glycerol dehydrogenases of Pseudomonas salinaria, Vibrio costicolus and Escherechia coli in relation to bacterial halophilism. Can. J. Physiol. Pharmacol. 32:206-217.

12. Bayley, S. T. and D. J. Kushner. 1964. The ribosomes of the extremely halophilic bacterium, Halobacterium cutirubrum. J. Mol. Biol. 9:654-669.

13. Beerens, H., S. Sugama and M. Tahon-Castel. 1965. Psychrotrophic Clostridia. J. Appl. Bacteriol. 28:36-48.

14. Berry, W. L. and W. W. Thomson. 1967. Composition of salt secreted by salt glands of Tamarix aphylla. Can. J. Bot. 45:1774-1775.

15. Biebl, R. 1939. Protoplasmstische Oekologie der Meeresalgen. Ber. Deut. Bot. Ges. 57:79-90.

16. Black, R. F. 1960. Effects of NaCl on the ion uptake and growth of Atriplex vesicaria Heward. Aust. J. Biol. Sci. 13:249-266.

17. Blum, H. F. 1933. Light responses in the brine flagellate D. salina with respect to wavelength. Univ. Calif. Publ. Physiol. 8:21-30.

18. Borgstrom, G. 1961. Unsolved problems in frozen food microbiology. Proceedlings of the Low Temperature Microbiology Symposium, Cambell Soup Co., pp. 197-250.

19. Brandts, John F., Joan Fu and John H. Nordin. 1970. The low temperature denaturation of chymotrypsinogen in aqueous solutions and in frozen aqueous solutions. In: G. E. W. Wolstenholme and Maeve O'Connor, eds. The Frozen Cell. J. and A. Churchill, London. pp. 189-209.

20. Britten, R. J. and F. T. McClure. 1960. The amino acid pool of E. coli. Bacteriol. Rev. 26:292-330.

21. Brown, A. D. 1963. The peripheral structures of gram-negative bacteria IV. The cation-sensitive dissolution of the cell membrane of the halophilic bacterium, Halobacterium halobium. Biochem. Biophys. Acta 75:425-435.

22. Brown, A. D. and C. D. Shorey. 1965. An alternative method of isolating the membrane of a halophilic bacterium. J. Gen. Microbiol. 41:225-231.

23. Burns, M. E. 1964. Cryobiology as viewed by the microbiologist. Cryobiology 1:18-39.

24. Butcher, R. W. 1959. An introductory account of the smaller algae of British coastal waters. Fish. Invest. Min. Agr. Fish. Food (Gt Brit) Ser. IV. pp. 21-24.

25. Chambers, R. and H. P. Hale. 1932. The formation of ice in protoplasm. Proc. Roy. Soc. London B Biol. Sci. 110: 236-352.

26. Chen, A. W. 1964. Soil fungi with high salt resistance. Trans. Kans. Acad. Sci. 67:36-40.

27. Cho, K. Y. and M. R. J. Salton. 1966. Fatty acid composition of bacterial membrane and wall lipids. Biochem. Biophys. Acta 116:73-79.

28. Chodat, R. 1922. Materiaux pour l'histoire des algues de la Suisse. Bull. Soc. Botan. Geneve 13:66-114.

29. Christian, J. H. B. and Judith A. Waltho. 1962. Solute concentrations within cells of halophilic and non-halophilic bacteria. Biochem. Biophys. Acta 65:506-508.

30. Connor, D. J. 1969. Growth of the grey mangrove, Avicennia marina in nutrient culture. Biotropica 1:36-40.

31. Craigie, J. S. and J. McLachlan. 1964. Glycerol as a photosynthetic product in Dunaliella tertiolecta Butcher. Can. J. Bot. 42:777-778.

32. Cowman, R. A. and M. L. Spock. 1969. Low temperature as an environmental stress on microbial enzymes. Cryobiology 5: 291-299.

33. Davies, Anthony G. 1970. Iron, chelation and the growth of marine phytoplankton. I. Growth kinetics and chlorophyll production in cultures of the euryhaline flagellate Dunaliella tertiolecta under iron limiting conditions. J. Mar. Biol. Ass. U. K. 50:65-86.

34. Davies, J. D. 1970. The role of peptides in preventing freeze-thaw injury. In: G. E. W. Wolstenholm and Maeve O'Connor, eds. The Frozen Cell. J. and A. Churchill, London. pp. 213-233.

35. DeVries, Arthur L. 1971. Glycoproteins as biological antifreeze agents in Antarctic fishes. Science (Washington) 172:1152-1155.

36. Dodds, J. J. A. and R. J. Ellis. 1966. Cation stimulated adenosine triphosphatase activity in plant cell walls. Biochem. J. 101:31.

37. Drovka, I. H., R. T. Popova and N. D. Tupyk. 1964. Carotene content in the alga Dunaliella salina when grown under laboratory conditions. Ukrayins'kyi Bot. Zh. 21:44-49.

38. Drovka, I. H. and S. I. Dovhoruka. 1966. Carotene formation in Dunaliella salina under the effect of some carbon sources. Ukrayins'kyi Bot. Zh. 23:59-62.

39. Drovka, I. H. and R. T. Popova. 1969. Pigment content of two strains of D. salina Teod. Ukrayins'kyi Bot. Zh. 26:90-92.

40. Drovka, I. H. and R. T. Popova. 1969. Carotene content in Dunaliella salina Teod. under mass culture conditions. Ukrayins'kyi Bot. Zh. 26:17-20.

41. Drovka, I. H. 1970. Some carotene containing strains of the alga Dunaliella salina Teod. Ukrayins'kyi Bot. Zh. 27:370-372.

42. Drovka, I. H. 1971. Stereoisomers of B-carotene of Dunaliella salina Teod. Ukrayins'kyi Bot. Zh. 28:670-673.

43. Eddy, B. P. 1960. The use and meaning of the term psychrophilic. J. Appl. Bacteriol. 23:189-190.

44. Elazari-Volcani, B. 1943. A Dimastibamoeba in the bed of the Dead Sea. Nature (London) 152:301-302.

45. Eppley, Richard W. 1962. Major cations. In: Ralph A. Lewin, ed. Physiology and Biochemistry of the Algae. Academic Press, New York and London. pp. 255-266.

46. Eppley, Richard W. and F. M. Maciasr. 1963. Temperature relationships in the growth of Dunaliella tertiolecta and its dependence upon salt concentration. Amer. J. Bot. 50:629-632.

47. Eppley, Richard W. and Phillip R. Sloan. 1965. Carbon balance experiments with marine phytoplankton. J. Fish. Res. Board Can. 22:1083-1097.

48. Farrant, J. 1970. Mechanisms of injury and protection in living cells and tissues at low temperatures. In: Audrey U. Smith, ed. Current Trends in Cryobiology. Plenum Press, New York and London. pp. 139-152.

49. Farrant, John and A. E. Woolgar. 1972. Human red cells under hypertonic stress; a model system for investigating freezing damage 2. Sucrose. Cryobiology 9:16-21.

50. Fedorov, V. D., V. N. Maksimov and V. M. Khromov. 1968. Effect of light and temperature on the primary production of some unicellular green algae and diatoms. Fizol. Rast. 15:640-651.

51. Fritch, R. E. 1935. The Structure and Reproduction of the Algae. Vol. 1. Cambridge University Press, London and New York.

52. Geiger, P. J., L. D. Jaffe and G. Mamikunian. 1965. Biological contamination of the planets. In: G. Mamikunian and M. H. Briggs, eds. Current Aspects of Exobiology. Pergamon Press New York.

53. Ginzburg, Margaret. 1969. The unusual permeability of two halophilic unicellular organisms. Biochim. Biophys. Acta 173:370-376.

54. Gorke, H. 1906. Uber den Kalletod der Pflanyen und seine Ursachen. Landw. Vers. Sta. 65:149-160.

55. Grant, B. R. 1968. The effect of carbon dioxide concentration and buffer system on nitrate and nitrite assimilation by Dunaliella tertiolecta. J. Gen Microbiol. 54:327-336.

56. Grant, Bruce R. 1970. Nitrite reductase in Dunaliella tertiolecta: isolation and properties. Plant. Cell. Physiol. 11:55-64.

57. Greenway, H., B. Klepper and P. G. Hughes. 1968. Effects of low water potential on ion uptake and loss for excised roots. Planta Arch. Wiss. Bot. 80:129-141.

58. Greiff, Donald and Richard T. Kelly. 1966. Cryotolerance of enzymes I. Freezing of lactic dehydrogenase. Cryobiology 2:335-341.

59. Guillard, R. R. L. 1960. A mutant of Chlamydomonas moewusii lacking contractile vacucles. J. Protozool. 7:262-268.

60. Heber, U. W. and K. A. Santarius. 1964. Loss of adenosine triphophate synthesis caused by freezing and its relationship to frost hardiness problems. Plant Physiol. 39: 712-719.

61. Heber, U. and K. A. Santarius. 1967. Biochemical and Physiological aspects of plant frost-resistance. In: A. S. Troshin ed. The Cell and Environmental Temperature. Pergamon Press, New York. pp.27-34.

62. Heber, U. 1970. Proteins capable of protecting chloroplast membranes against freezing. In: G. E. W. Wolstenholme and Maeve O'Connor. eds. The Frozen Cell. J. and A. Churchill, London. pp. 175-186.

63. Heleskul, Y. F. 1964. Chromatographic and spectrophotometric investigation of the carotenoids of the alga Dunaliella salina and the determination of their biological activity. Ukrayins'kyi Biochim. h. 36:778-783.

64. Hellebust, Johen A. and John Terborgh. 1967. Effects of environmental conditions on the rate of photosynthesis and some photosynthetic enzymens in Dunaliella tertiolecta Butcher. Limnol. Oceanogr. 12:559-567.

65. Hof, T. and P. Fremy. 1933. On Myxophyceae living in strong brines. Rec. Trav. Bot. Neerland 30:140-162.

66. Holmes, P. K. and H. O. Halvorson. 1965. Purification of a salt requiring enzyme from an obligately halophilic bacterium. J. Bacteriol. 90:312-315.

67. Holm-Hansen, O. 1963. Viability of bluegreen and green algae after freezing. Physiol. Plant. 16:530-540.

68. Horowitz, N. H., Roy E. Cameron, and Jerry S. Hubbard. 1972. Microbiology of the Dry Valleys of Antarctica: Studies in the worlds coldest and driest desert have implications for the Mars biological program. Science 176:242-245.

69. Hutner, S. F., L. Provasoli, Albert Schatz, and C. P. Haskins 1950. Some approaches to the study of the role of metals in the metabolism of microorganisms. Proceedings of the American Philosophical Society 94 No. 2 pp. 152-170.

70. Hwang, Shush-Wei and Wanda Horneland. 1965. Survival of algal cultures after freezing by controlled and uncontrolled cooling. Cryobiology 5:305-311.

71. Iljin, W. S. 1933. Protoplasma 20:105-124.

72. Ingraham, J. L. and J. L. Stokes. 1959. Psychrophilic bacteria. Bacteriol. Rev. 23:97-108.

73. Ingraham, John L. 1969. Factors which preclude growth of bacteria at low temperature. Cryobiology 6:188-193.

74. Johnson, F. H. 1954. The Kinetic Basis of Molecular Biology. Wiley, New York.

75. Jokela, Chung-Chau Tang. 1970. Outer membrane of Dunaliella tertiolecta: isolation and properties. Diss. Abstr. Int B Sci. Eng. 30:5122.

76. Kanwisher, J. W. 1955. Freezing in intertidal animals. Biol. Bull. (WoodsHole) 109:56-63.

77. Kanwisher, John. 1957. Freezing and drying in intertidal algae. Biol. Bull. (Woods Hole) 113:275-285.

78. Kappen, Von Ludger. 1969. Frostresistenz einheimischer Halophyten in Beziehung zu ihrem Salz-, Zucker-, and Wassergehalt im Sommer und Winter. Flora allg. Bot. Ztg. (Jena) 158:232-260.

79. Kappen, Von Ludger. 1969. Kaltvertraglichkeit und Zuckergehalt von Salzpflazen. Ber. Deut. Bot. Ges. 82:103-106.

80. Kappen, Von Ludger and Oto L. Lange. 1972. Die Kalter Resistenz einger Makrolichenen. Flora Allg. Bot. Ztg. (Jena) 161:1-29.

81. Karush, Fred, Norman R. Kliman and Robert Marks. 1963. An assay method for disulfide groups by fluorescense quenching Anal. Biochem. 9:100-114.

82. Kiener, Walter. 1944. Green snow in Nebraska. Proc. Nebraska Acad. Sci. Affiliated Soc. 54:12.

83. Kessler, B., N. Engelberg, D. Chen and H. Greenspan. 1964. Studies on physiological and biochemical problems of stress in higher plants. Spec. Bul. Isr. Ministry Agr. Rehovot. 64:74-81.

84. Kleinschmidt, M. G. and Vern McMahon. 1970. Effect of growth temperature on the lipid concentration of Cyanidium caldarium. I. Class separation of lipids. Plant Physiol. 46:286-289.

85. Kleinschmidt, M. G. and Vern McMahon. 1970. Effect of growth temperature on the lipid composition of Cyanidium caldarium II. Glycolipid and phospholipid components. Plant Physiol. 46:290-293.

86. Krishna, P. V. 1955. Observations on the ionic composition of blue green algae growing in saline lagoons. Proc. Nat. Inst. Sci. India Part B, Biol. Sci. 21:90-102.

87. Kuiper, Pieter J. C. 1964. Inducing resistance to freezing and dessication in plants by decenylsuccinic acid. Science 146: 544-546.

88. Kylin, A, and R, Gee. 1970. Adenosine triphosphate activities in leaves of the mangrove Avicennia nitrida Jacq. Influence of sodium to potassium ratios and salt concentrations. Plant Physiol. 45:169-172.

89. Kwon, Y. M. and B. R. Grant. 1971. Assimilation and metabolism by cell free systems. Plant Cell. Physiol. 12:29-39.

90. Lagerheim, G. 1892. Die Schneeflora des Pichincha. Ber. Deut. Bot. Ges. 10:517-534.

91. Larsen, Helge. 1967. Biochemical aspects of extreme halophilism. In: A. H. Rose and J. F. Wilkinson, eds. Advances in Mycrobial Physiollogy, Vol. 1. Academic Press, London and New York. pp. 97-132.

92. Levitt, J. 1964. Cryobiology as viewed by a botanist. Cryobiology 1:11-17.

93. Levitt, J. 1965. Thiogel-a model system for demonstrating intermolecular disulfide bond formation on freezing. Cryobiology 1:312-316.

94. Levitt, J. 1966. Winter hardiness in plants. In: H. T. Meryman, ed. Cryobiology Academic Press, New York and London. pp. 495-563.

95. Levitt, J. 1966. Cryochemistry of plant tissue protein interactions. Cryobiology 3:243-251.

96. Levitt, J. and John Dear. 1970. The role of membrane proteins in freezing injury and resistance. In: G. E. W. Wolstenholme and Maeve O'Connor, eds. The Frozen Cell. J. and A. Churchill, London. pp. 149-173.

97. Levitt, J. 1972. Responses of Plants to Environmental Stresses Academic Press, New York and London.

98. Lewin, Ralph A. 1962. Physiology and Biochemistry of the Algae Academic Press, New York. pp. 541-555.

99. Luyet, B. J. 1970. Physical changes occuring in frozen solutions during rewarming and melting. In: G. E. W. Wolstenholme and Maeve O'Connor, eds. The Frozen Cell. J. and A. Churchill, London. pp. 27-64.

100. MacKenzie, A. P. 1970. Death of frozen yeast in the course of slow warming. In: G. E. W. Wolstenholme and Maeve O'Connor eds. The Frozen Cell. J. and A. Churchill, London. pp. 89-114.

101. Madgwick, J. C. 1965. Quantitative chromatography of algal chlorophylls (Nitzschia closterium and Dunaliella tertiolecta) on thin layers of glucose. Deep-Sea Res. Oceanogr. Abstr. 12:233-236.

102. Marre, E. and O. Servattaz. 1959. Sul meccanismo di adattamento a condizioni osmotische estreme. II. Concentrazione del mezzo esterno e composizione del succo cellulare. Atti acad. nazl. Loncei Rend. Class sci. fis. mat. e nat. 26:272-278.

103. Masyuk, N. P. 1961. Carotene producing alga Dunaliella salina Teod. in salt water reservoirs of the Krymsk Oblast. Ukrayins'kyi Bot. Zh. 18:100-109.

104. Masyuk, N. P. 1965. Carbonates and bicarbonates as stimulators of growth and carotene production in Dunaliella salina culture. Ukrayins'kyi Bot. Zh. 22:18-22.

105. Masyuk, N. P. 1966. Mass culture of the carotene-bearing alga Dunaliella salina. Ukrayins'kyi Bot. Zh. 23:12-19.

106. Masyuk, N. P. and Y. H. Abdulla. 1969. First experiment of growing carotene algae under semi-industrial conditions.

107. Masyuk, N. P., Y. H. Abdulla and M. I. Radchenko. 1970. Effect of some outside organisms on culture of Dunaliella salina Teod. under semi-industrial conditions. Ukrayins' kyi Bot. Zh. 27:456-461.

108. Mazur, P. 1961. Physical and temporal factors involved in the death of yeast at sub-zero temperatures. Biophys. J. 1:247-264.

109. Mazur, Peter. 1963. Kinetics of water loss from cells brought to sub-zero temperatures and the likelihood of intracellular freezing. J. Can. Physiol. 47:347-368.

110. Mazur, Peter. 1966. Physical and chemical basis of injury in single celled micro-organisms subjected to freezing and thawing. In: H. T. Meryman, ed. Cryobiology. Academic Press, New York. pp. 214-315.

111. Mazur, Peter and Janice Schmidt. 1968. Interactions of cooling velocity, temperature, and warming velocity on the survival of frozen and thawed yeast. Cryobiology 5:1-17.

112. Mazur, Peter. 1970. Cryobiology: the freezing of biological systems. Science (Washington) 168:939-949.

113. Mazur, P., S. P. Leibo and E. H. Y. Chu. 1972. The two-factor hypothesis of freezing injury. Evidence from Chinese hamster tissue culture cells. Exp. Cell Res. 71:345-355.

114. McLachlan, Jack. 1960. The culture of Dunaliella tertiolecta Butcher - a euryhaline organism. Can. J. Microbiol. 6:367-379.

115. Meryman, Harold T. 1966. Review of biological freezing. In: Harold T. Meryman, ed. Cryobiology. Academic Press. London and New York. pp. 2-81.

116. Meryman, H. T. 1971. Cryoprotective agents. Cryobiology 8:173-183.

117. Meyer, George H., Marie B. Marrow, and Orville Wyss. 1962. Antarctica: the microbiology of an unfrozen saline pond. Science 138:1103-1104.

118. Mil'ko, E. S. 1963. Effect of illumination and temperature on pigment formation in Dunaliella salina. Mikrobiologiya 32:590-597.

119. Mironyuk, V. I. and L. O. Einor. 1968. Oxygen exchange and the content of pigments in various forms of Dunaliella salina Teod. under conditions of increased NaCl content. Gidrobiol. Zh. 4:23-29.

120. Mironyuk, V. I. 1969. Some properties of the oxidation-reduction systems of the unicellular alga Dunaliella salina Teod. Ukrayins'kyi Bot. Zh. 26:54-59.

121. Mironyuk, V. I. 1969. Catalase and peroxidase of Dunaliella salina Teod. Ukrayins'kyi Bot. Zh. 26:92-95.

122. Mironyuk, V. I. and L. O. Einor. 1970. Effect of phenol derivatives on the oxygen metabolism of Dunaliella salina Teod. Gidrobiol. Zh. 6:91-95.

123. Mohr, V. and H. Larsen. 1963. On the structural transformations and lysis of Halobacterium salinarium in hypotonic and isotonic solutions. J. Gen. Microbiol. 31:267-280.

124. Mushyak, P. O. 1968. Content and state of nucleic acids in the alga Dunaliella salina Teod. depending on salt concentrations. Ukrayins'kyi Bot. Zh. 25:91-95.

125. Nei, Tokio. 1970. Mechanism of haemolysis of erythrocytes by freezing, with special reference to freezing at near zero temperature. In: G. E. W. Wolstenholme and Maeve O'Connor, eds. The Frozen Cell. J. and A. Churchill, London. pp. 13-142.

126. Nemethy, George. 1966. Structure of water and of aqueous solutions. Cryobiology 3:19-26.

127. Norberg, P. and B. V. Hofsten. 1970. Chromatography of a halophilic enzyme on hydroxyapatite in 3.4 M sodium chloride. Biochem. Biophys. Acta 220:132-133.

128. Nordin, J. H., Richard Duffield, Nancy Freedman, William Gelb and J. R. Brandts. 1970. Enzyme activity in cryobiological systems. Studies on glycolytic enzymes and low temperature-induced accumulation in Hymenoptera. Cryobiology 6:373-384.

129. Norkrans, B. 1968. Studies on marine occurring yeasts: respiration, fermentation and salt tolerance. Arch. Mikrobiol. 62:358-372.

130. Olsen, R. H. 1967. Isolation and growth of psychrophilic bacteriophage. Appl. Microbiol. 15:198-200.

131. Onishi, H. 1963. Studies on osmophilic yeasts. XV. The effects of high concentrations of NaCl on polyalcohol production. Agri. Biol. Chem. 27:543-545.

132. Osmond, C. B. 1967. Acid metabolism in Atriplex. I.Regulation of oxalate synthesis by the apparent excess cation absorption of leaf tissue. Aust. J. Biol. Sci. 20:575-587.

133. Paasche, E. 1971. Effect of ammonia and nitrate on growth photosynthesis, and ribulose-diphosphate carboxylase content of Dunaliella tertiolecta. Physiol. Plant. 25:294-299.

134. Penn, Amos Benkoo and Kuan Chin. 1937. Die Cytologie der Zelltheilung von Dunaliella (Teodoresco). Arch. Protistenk 90:162-164.

135. Peridsky, M. D. and M. H. Ellett. 1971. Lysosomes and cell cryoinjury. Cryobiology 8:345-349.

136. Pochman, A. 1959. Uber die Tatigkeit der nichtkontraktilen Importvacuole und den Modus der Osmoregulation bei dem Salzflagellaten Choanogaster nevst Bemerkungen uber die Funktion der Posulen. Ber Deut. Bot. Ges. 72:99-108.

137. Post, R. L. 1959. Relationship of an ATP-ase in human erythrocyte membrane to the active transport of sodium and potassium. Fed. Proc. 18:121-123.

138. Radchenko, M. L. and N. P. Masyuk. 1969. Chromatographic methods for the study of the pigments of polyblepharid algae. Gidrobiol. Zh. 5:130-132.

139. Repp, G. 1958. Die Salztoleranz der Pflanzen. I. Salzhaushalt und Salzresistenz von Marschpflanzen der Nordseekustr Danemarks in Beziehung zum Standort. Oesterr. Bot. Z. 104:454-490.

140. Rice, F. O. 1960. In: A. M. Bass and H. P. Broida, eds. Formation and Trapping of Free Radicals. Academic Press, New York. pp. 7-30.

141. Sakai, A. 1961. Effect of polyhydric alcohols on frost hardiness in plants. Nature (London) 189:416-417.

142. Salton, M. R. J. 1964. The Bacterial Cell Wall. Elsevier, Amsterdam.

143. Scarth, G. W. 1936. Trans. Roy. Soc. Can. 30:1-10.

144. Scholander, P. F. 1957. Supercooling and osmoregulation in Arctic fish. J. Cell. Physiol. 49:5-24.

145. Scholander, P. F., H. R. Hammel, E. Hemmingsen and W. Garey. 1962. Salt balance in mangroves. Plant Physiol. 37:722-729.

146. Scott, G. T. and H. R. Haywood. 1953. Metabolic factors influencing the sodium and potassium distribution in Ulva lactuca. J. Gen Physiol. 36:659-671.

147. Sehgal, S. N., M. Kates and N. E. Gibbons. 1962. Lipids of Halobacterium cutirubrum. Cand. J. Biochem. 40:69-80.

148. Shevyokova, N. I. and E. I. Komiyerko. 1969. Amino acid metabolism in culture of callus tissue from cabbage leaves (Brassica oleraceae va. capitata) under salinization conditions. Dokl. Akad. Nauk. SSSR Bot. Sci. Sect. (Transl.) 186:1441-1444.

149. Siegel, S. M., K. Roberts, M. Lederman and O. Daly. 1967. Microbiology of saturated salt solutions and other harsh environments. II. Ribonucleotide dependency in the growth of a salt-habituated Penicillium notatum in salt-free media. Plant Physiol. 42:201-204.

150. Siegel, S. M. 1968. Elements of space biology. Advan. Space Sci. Technol. 9:1-100.

151. Siegel, S. M., Henry C. Nathan, and Karen Roberts. 1968. Experimental biology of ammonia-rich environments: optical and isotopic evidence for vital activity in Penicillium in liquid ammonia-glucerol media at -40°C. Proc. Nat. Aca. Sci. U. S. A. 60:505-508.

152. Siegel, S. M. 1969. Microbiology of saturated salt solutions and other harsh environments. V. Relation of inosine-5-phosphate and carbohydrate to growth of wildtype and mutant Penicillium in boric acid and potassium chloride selective media. Physiol. Plant. 22:1152-1157.

153. Siegel, S. M., Thomas Speitel and Roy Stoecker. 1969. Life in earth extreme environments: a study in cryobiotic potentialities. Cryobiology 6:160-181.

154. Siegel, S, M. 1973. Solubilization and accumulation of copper from elementary surfaces by Penicillium notatum. Environmen. Bio. Med. 2:19-22.

155. Simon, Ellen M. 1971. Paramecium aurelia: recovery from -196°C Cryobiology 8:361-365.

156. Skinner, F. A. 1968. The limits of microbial existence. Proc. Roy. Soc. London B Biol. Sci. 171:77-99.

157. Smith, G. M. 1951. Manual of Phycology. Ronald Press, New York.

158. Stroganov, B. P. and L. P. Lapina. 1964. A possible method for the separate study of toxic and osmotic action of salts on plants. Fiziol. Rast. 11:674-680.

159. Subba Rao, D. V. and T. Platt. 1969. Optimal extraction conditions for chlorophylls from cultures of five species of marine phytoplankton. J. Fish. Res. Board Can. 26:1625-1630.

160. Thomson, W. W., W. L. Berry and L. L. Liu. 1969. Localization and secretion of salt by the salt gland of Tamrix aphylla. Proc. Nat. Acad. Sci. U. S. A. 63:310-317.

161. Trezzi, F. M., M. G. Galli and E. Bellini. 1965. L'osmoresistenza di Dunaliella salina ricerche ultrastruttarali. G. Bot. Ital. 72:255-263.

162. Ungar, I. A. 1962. The influence of salinity on seed germination in succulent plants. Ecology 43:763-764.

163. Wallen. D. G. and G. H. Geen. 1971. Light quality and concentration of proteins, RNA, DNA, and photosynthetic pigments in two species of marine plankton algae. Mar. Biol. (Berlin) 10:44-51.

164. Wegman, Klaus. 1971. Osmotic regulation of photosynthetic glycerol production in Dunaliella. Biochem. Biophys. Acta 234:317-323.

165. Whittingham, C. G., S. P. Leibo and P. Mazur. 1972. Survival of mouse embryos frozen to -196°C. Science (Washington) 178:411-414.

166. Wilce, R. T. 1960. Heterotrophy in Arctic Sublittoral Seaweeds: An Hypothesis. Bot. Mar. 10:185-197.

167. Winter, J. E. 1969. On the influence of food concentration and other factors on filtration rate and food utilization in the mussels Antarctica islandica and Modiolus modiolus. Mar. Biol. (Berlin) 4:87-135.

168. Wolfson, C. 1935. Observations of Paramecium during exposure to subzero temperatures. Ecology 16:630-639.

169. Yabe, A., G. Kuse, T. Maruta and H. Takada. 1965. Physiology of the halophytes. I. The osmotic value of leaves and the osmotic role of each ion in the cell sap. Physiol. Ecol. 13:25-33.

170. Yurina, E. V. 1966. Experiment in the cultivation of the halobiont algae Asteromonas gracilis and Dunaliella salina. Vestn. Mosk. Univ. 21:76-83.

171. Yurkova, H. N. 1965. Effect of the temperature factor on Dunaliella salina. Ukrayins'kyi Bot. Zh. 22:51-57.

172. Zanco, Fanny Fontana. 1931. Rapproti fra concentrazioni degli idrogenioni e possibilita di vita di organismi animalie vetetali nei bacini dell Saline di Cagliari. Publ. Staz. Biol. San Bartolomeo (Cagliari) 32:3-28.

173. ZoBell, C. E. 1957. Barophilic bacteria in some deep sea sediments. J. Bacteriol. 73:563-568.